

Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase in Chicken Embryo Fibroblasts

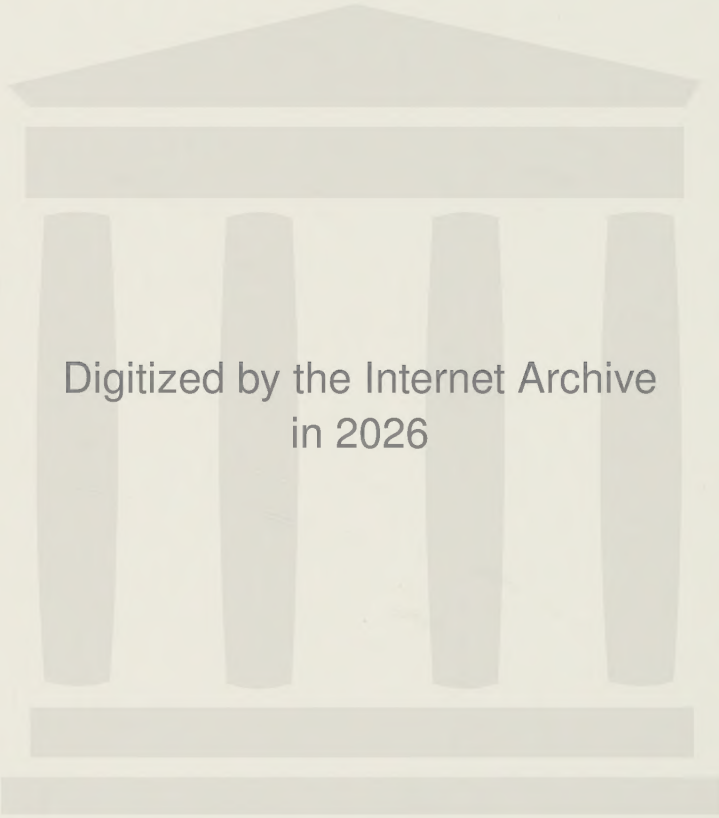
INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der
Universität Zürich

von
JÖRG FLÜCKIGER
aus Huttwil (BE)

Begutachtet von Herrn Prof. Dr. Ph. Christen

Zürich 1987
Zentralstelle der Studentenschaft



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Intracellular Location of the Precursor of Mitochondrial Aspartate Aminotransferase and Its in vitro Import into Mitochondria. Flückiger, J., Behra, R., Jaussi, R., and Christen, P. (1982) 14th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Interlaken, 1982, Experientia 38, 740

Inhibitors of Maturation and Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase. Jaussi, R., Flückiger, J., Behra, R., and Christen, P. (1984) in 'Chemical and Biological Aspects of Vitamin B₆ Catalysis', Part B (Evangelopoulos, A.E., ed) pp. 27-37, Alan R. Liss, New York

Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase in Chicken Embryo Fibroblasts. Flückiger, J., and Christen, P. (1984) 16th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Zürich, 1984, Experientia 40, 623

Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase in Chicken Embryo Fibroblasts. Flückiger, J., and Christen, P. (1986) 19th Meeting of the Federation of European Biochemical Societies (FEBS), Berlin West, 1986, Biol. Chem. Hoppe-Seyler 367, 340

Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase in Chicken Embryo Fibroblasts. Flückiger, J., and Christen, P. (1987) 19th Meeting of the Swiss Societies for Experimental Biology (USGEB), Lausanne, 1987

Genes, Biosynthesis, and Intracellular Processing of the Isoenzymes of Aspartate Aminotransferase. Christen, P., Jaussi, R., Gehring, H., Sharma, C.P., Flückiger, J., Juretić, N., Flura, T., Mattes, U., and Lindenmann, J.-M. (1987) International Congress on Chemical and Biological Aspects of Vitamin B₆ Catalysis, Turku, Finland, 1987, Abstracts

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Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase in Chicken Embryo Fibroblasts. Flückiger, J., and Christen, P.

Abbreviations

mAspAT	Mitochondrial aspartate aminotransferase
pre-mAspAT	Precursor of mAspAT
CEF	Chicken embryo fibroblasts
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
BME	Basal Medium Eagle
FCS	Fetal calf serum
SDS	Sodium dodecyl sulfate
dGlc	2-Deoxyglucose

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SUMMARY

The precursor of mitochondrial aspartate aminotransferase accumulates under conditions of import block in the cytosol of cultured chicken embryo fibroblasts (CEF). However, its accumulation is limited by degradation with a half-life of only ~5 min (Jaussi, R., Sonderegger, P., Flückiger, J., and Christen, P. (1982) *J. Biol. Chem.* 257, 13334-13340). The aim of the present study was the characterization of the proteolytic systems responsible for this very rapid intracellular degradation. On depleting CEF of ATP, the rate of degradation of the precursor was lowered by ~70 %. CEF depleted of divalent metal ions showed a degradative activity of 10 % of the initial value. Reconstitution of these cells with Mg^{2+} and Ca^{2+} increased the degradative activity from 10 to 107 and 24 %, respectively. Thiol reagents almost completely prevented the degradation whereas specific peptide inhibitors of cysteine proteases or inhibitors of intralysosomal proteolysis decreased the rate of degradation by only ~30 %. Inhibitors of serine proteases had little effect. No rapid degradation of the precursor was observed in crude extracts of CEF. The data indicate that the bulk of the precursor accumulated under conditions of import block is degraded by one or several cytosolic proteases dependent on ATP, Mg^{2+} , and thiol groups of unknown localization, conceivably by proteolytic enzymes identical with or similar to one of the high molecular weight cytosolic proteases (Waxman, L., Fagan, J.M., Tanaka, K., and Goldberg, A.L. (1985) *J. Biol. Chem.* 260, 11994-12000). The rest of the precursor appears to be degraded by lysosomes.

ZUSAMMENFASSUNG

Der Vorläufer der mitochondrialen Aspartat-Aminotransferase akkumuliert bei Importhemmung im Zytosol von kultivierten Hühnerembryofibroblasten. Seine Akkumulation ist hingegen durch Degradation mit einer Halbwertszeit von ~ 5 min begrenzt (Jaussi, R., Sonderegger, P., Flückiger, J., and Christen, P. (1982) J. Biol. Chem. 257, 13334-13340). Das Ziel der vorliegenden Studie war die Charakterisierung der proteolytischen Systeme, welche für diese sehr schnelle intrazelluläre Degradation verantwortlich sind. Wenn Hühnerembryofibroblasten an ATP erschöpft wurden, nahm die Rate der Vorläuferdegradation um ~ 70 % ab. An zweiwertigen Metallionen erschöpfte Hühnerembryofibroblasten zeigten eine Degradationsaktivität von 10 % des Anfangswertes. Rekonstitution dieser Zellen mit Mg^{2+} beziehungsweise Ca^{2+} erhöhte die Degradationsaktivität von 10 auf 107 beziehungsweise 24 %. Thiolreagentien verhinderten die Degradation fast vollständig, während spezifische Peptidinhibitoren von Cysteinproteasen oder Inhibitoren der intralysosomalen Proteolyse die Degradationsrate um nur ~ 30 % erniedrigten. Inhibitoren von Serinproteasen hatten wenig Wirkung. Keine schnelle Degradation des Vorläufers wurde in Rohextrakten von Hühnerembryofibroblasten beobachtet. Wir schliessen daraus, dass der Grossteil des bei Importhemmung akkumulierten Vorläufers durch eine oder mehrere zytosolische Proteasen degradiert wird, die von ATP, Mg^{2+} und Thiolgruppen unbekannter Lokalisierung abhängen, wobei denkbar ist, dass mit den hochmolekularen zytosolischen Proteasen identische oder ihnen ähnliche proteolytische Enzyme beteiligt sind (Waxman, L., Fagan, J.M., Tanaka, K., and Goldberg, A.L. (1985) J. Biol. Chem. 260, 11994-12000). Der Rest des Vorläufers scheint durch Lysosomen degradiert zu werden.

1. INTRODUCTION

The precursor of mitochondrial aspartate aminotransferase (pre-mAspAT) is coded for by nuclear DNA and synthesized with an NH₂-terminal extrapeptide on free polysomes in the cytosol (Sonderegger et al., 1982). In cultured chicken embryo fibroblasts (CEF) the post-translational import of the precursor into the mitochondrial matrix proceeds with a half-life of 0.5 - 1 min (Jaussi et al., 1982). It can be completely inhibited by treating the cells with reagents that dissipate the mitochondrial membrane potential such as the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP; Jaussi et al., 1982) or the ionophore valinomycin (unpublished results). Under these conditions, the preprotein accumulates in the cytosol of CEF. However, rapid degradation ($t_{1/2} \sim 5$ min) limits the increase in the steady state concentration of pre-mAspAT to one order of magnitude (Jaussi et al., 1982). The high susceptibility of the precursor to proteolytic attack by intracellular proteases contrasts the in vivo stability of the mature enzyme ($t_{1/2} \sim 3$ days or more, see Shrawder and Martinez-Carrion, 1973; Shaffer and Felder, 1983; Sharma and Gehring, submitted). Accordingly, exogenous proteases rapidly degrade pre-mAspAT (Jaussi et al., 1982) in vitro whereas mAspAT proves remarkably stable (Sandmeier and Christen, 1980), indicating differences in the conformation or quaternary structure of the two proteins.

The half-lives of precursors of different mitochondrial matrix and inner membrane proteins accumulated under inhibition of uptake or processing vary greatly; in all cases, however, they are much shorter than the average half-life of total mitochondrial proteins ($t_{1/2} = 6.8$ days, see Glaumann et al., 1981). Degradation rates similar to that of pre-mAspAT were reported for pre-carbamoyl phosphate synthetase in rat liver explants

(Raymond and Shore, 1981) and isolated rat hepatocytes (Morita et al., 1982), pre-ornithine carbamoyltransferase in isolated rat hepatocytes (Morita et al., 1982), pre-cytochrome c_1 in hepatoma cells (Kolarov and Nelson, 1984) and yeast (Reid and Schatz, 1982), and for the pre-iron sulfur protein of the cytochrome $b\cdot c_1$ complex in hepatoma cells (Kolarov and Nelson, 1984). Pre- δ -aminolaevulinate synthase is degraded in hepatocytes of embryonic chicken with a half-life of 30 min (Ades et al., 1983) whereas pre-methylmalonyl-CoA mutase in isolated rat liver cells (Fenton et al., 1984), as well as the precursor of the β -subunit of the F_1 -ATPase in hepatoma (Kolarov and Hatalová, 1984) and yeast cells (Reid and Schatz, 1982) show remarkable catabolic stability with half-lives in the range of hours. In none of these reports, the proteolytic systems acting on the precursors were studied.

Conceivably, the availability of mitochondrial proteins modulates mitochondriogenesis. The size of the cytosolic pools of nuclear-encoded mitochondrial preproteins is determined by the rates not only of their synthesis and import, but also of their degradation. Thus, degradation of mitochondrial preproteins could be instrumental to the regulation of the relative quantities of individual components being taken up by mitochondria. In view of the very scanty knowledge of the molecular events involved in these degradative processes, the present work was aimed at characterizing the proteases responsible for the degradation of pre-mAspAT in the cytosol of cultured CEF.

2. EXPERIMENTAL PROCEDURES

2.1 Materials

Basal Medium Eagle (BME) and fetal calf serum (FCS) were obtained from Gibco. BME prepared without methionine or without Ca^{2+} and Mg^{2+} was from Amimed; [^{35}S] methionine (~ 1000 Ci mmol^{-1}) from New England Nuclear; sodium dodecyl sulfate (SDS) and sodium orthovanadate from British Drug House. The chemicals for polyacrylamide gel electrophoresis, 2-deoxyglucose (d3lc), and digitonin were from Serva. Leupeptin, antipain, chymostatin, pepstatin, and bestatin were obtained from Peptide Institute, Osaka, Japan. Aprotinin (TrasylolTM) was from Bayer; E-64 from Boehringer Mannheim; colchicine from Sandoz; podophyllotoxin from Janssen; A23187 from Calbiochem, and magnesium chloride from Mallinckrodt. Calcium chloride, zinc chloride, and methylammonium chloride were from Merck. Fluka supplied ETH 1001 (listed as "calcium ionophore"). Monensin, quercetin, iodoacetamide, N-ethylmaleimide, p-hydroxymercuribenzoate, phenylmethylsulfonyl fluoride, p-aminobenzamidine, and bacitracin were from Sigma; rhodamine 123 as well as rhodamine B from Kodak. CCCP was a gift from Dr. P. G. Heytler, DuPont.

2.2 Cell Culture

CEF were grown in BME supplemented with Earle's salts and 10 % fetal calf serum (FCS). 100 mm-Petri dishes were used. The cultures were incubated at 37°C in a 95% air/5% CO_2 atmosphere.

2.3 Pulse-chase Experiments and Determination of the Rate of Protein Synthesis

Growing tertiary cultures of CEF at high density ($\sim 10^7$ cells per 100 mm-Petri dish) were washed for 7 min with 3 ml BME prepared without methionine. In order to accumulate labeled pre-mAspAT, the methionine-free medium was replaced by medium containing 40 μM CCCP and 0.02-0.13 mCi ml^{-1} [^{35}S]methionine ($\sim 1000 \text{ Ci mmol}^{-1}$). An incubation of 15 min was followed by a chase with 10 mM unlabeled methionine in the presence of 40 μM CCCP. CCCP was included in all pulse and chase media except those for the experiments with rhodamines. The duration of the chase periods for determining the half-life of pre-mAspAT ranged from 9 to 16 min. Additional ingredients are indicated in "Results". Unless otherwise specified, FCS was omitted from pulse-chase media. During all steps of the experiments, the Petri dishes were subjected to continuous tilting movements (10 min^{-1}). The chase was stopped by sucking off the medium, chilling the cultures on iced water, and immediately adding 100 μl of a mixture of pepstatin, leupeptin, antipain, chymostatin (0.8 mg ml^{-1} each), aprotinin (1.8 mg ml^{-1}), and EDTA (18 mM). After harvesting the cells at 0-4°C with a rubber policeman, the samples were denatured in boiling 1% SDS (final volume 800 μl).

The rate of protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable cellular proteins.

2.4 Isolation of pre-mAspAT

SDS-denatured samples were diluted to 0.1% SDS with Triton buffer and mAspAT was quantitatively immunoprecipitated in two

steps using monospecific antisera against SDS-denatured mAspAT (Sonderegger et al., 1982) in combination with glutaraldehyde-fixed Staphylococcus aureus (Cowan strain I) cells.

2.5 Electrophoresis and Fluorography

SDS-polyacrylamide gel electrophoresis was performed in 10% gel slabs. The gels were prepared for fluorography with the fluorescence enhancer EnlightningTM (New England Nuclear) according to the directions of the supplier and Fuji RX films were exposed for 1-5 days at -70°C.

2.6 Determination of the Rate of pre-mAspAT Degradation

For quantitation of the radioactivity in pre-mAspAT bands, the respective regions were cut out from the gel and eluted with the Lumasolve/Lipoluma system (Lumac) as recommended by the manufacturers. Alternatively, the fluorograms were subjected to scanning densitometry. The half-life of pre-mAspAT was calculated from the slopes of regression lines in semilogarithmic plots (see Fig. 2).

2.7 Determination of Degradation Rates of Long-Lived Cellular Proteins

The culture medium of growing tertiary CEF at high density was removed and 5 ml of BME containing 10% FCS and 10 μ Ci [³⁵S]methionine were added per Petri dish. After labeling for 16 h at 37°C, the cells were rinsed with 5 ml BME/1 mM unlabeled methionine/10 % FCS and then washed three times for 20 min with 7 ml of the same solution. Subsequently, the cells

were rinsed with the different degradation media (BME/1 mM methionine, supplemented with FCS and inhibitors of intracellular proteolysis as indicated in "Results"). The cells were then incubated in the degradation media for 5 h, and the rates of breakdown of long-lived proteins were determined during 4 h starting with the second hour of the incubation. Washes and the chases in the degradation media were performed under continuous tilting of the cultures (10 min^{-1}). After having collected the degradation media, both media and cells were chilled on iced water. The cells, mechanically harvested at $0-4^{\circ}\text{C}$, as well as the degradation media were subjected to precipitation with 10 % trichloroacetic acid/10 mM methionine in a total volume of 0.5 ml. Trichloroacetic acid-insoluble material was separated by centrifugation (10 min, $10,000 \times g_{\text{max}}$, 22°C), washed once with 1 ml 10 % trichloroacetic acid/10 mM methionine, and solubilized with 1 ml Lumasolve overnight at room temperature. Trichloroacetic acid-insoluble and trichloroacetic acid-soluble radioactivity was measured by scintillation counting. The degradation of long-lived cellular proteins was estimated by comparing the amount of extracellular trichloroacetic acid-soluble radioactivity released during the 4 h-chase period with the total amount of radioactivity present in long-lived proteins at the beginning of this period. If CEF were treated as described, intracellular trichloroacetic acid-soluble radioactivity remained small and constant after the three washing steps, indicating that the trichloroacetic acid-soluble radioactivity found extracellularly at the end of the degradation period was not due to $[^{35}\text{S}]$ methionine not removed from the cells by the washing procedure. Cell death and hence release of trichloroacetic acid-soluble radioactivity by bulk degradation in dying cells was shown to be negligible by staining control cultures with trypan blue at the beginning and at the end of the degradation period.

2.8 Determination of Intracellular ATP

In order to denature and precipitate cellular components rapidly, 3 ml of ice-cold 0.26 N perchloric acid were added per Petri dish of mock-pulsed and chased tertiary cells. The samples were harvested with a rubber policeman and put on ice for 30 min. After centrifugation (10 min, 20,000 x $g_{\max}$, 0°C), 100 μ l of the supernatants were added to 1.9 ml 100 mM Hepes/NaOH (pH 8.0) giving a pH value of 7.7, put on ice for another 30 min, and centrifuged as above. In the supernatants from the second centrifugation, ATP was determined with a test combination based on luciferin and luciferase from the American firefly (ATP bioluminescence CLS, Boehringer Mannheim). The luminescence signals were recorded with a LKB luminometer 1250.

3. RESULTS

3.1 Effect of Inhibitors of Intralysosomal Proteolysis on the Degradation of pre-mAspAT

Lysosomotropic weak bases such as methylamine rapidly elevate the pH value of acidic cellular vacuoles to nearly neutral levels (Maxfield, 1982). Consequently, protein breakdown by lysosomes is strongly and selectively inhibited; direct interactions of lysosomotropic weak bases with proteases were only observed in the case of aromatic amines (for a recent review, see Seglen, 1983). Monensin is a member of the group of carboxylic ionophores with broad specificity for monovalent cations used to increase the intravacuolar pH value, resembling lysosomotropic weak bases in this respect (Maxfield, 1982; Seglen, 1983).

In order to accumulate labeled pre-mAspAT, growing CEF were pulsed with [^{35}S]methionine in the presence of CCCP. The following chase was performed with unlabeled methionine in the presence of either CCCP alone, or CCCP plus 10 mM methylamine, or CCCP plus 30 μM monensin. After harvesting and denaturing the cells, pre-mAspAT was immunoprecipitated. The precipitates were analyzed with SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 1). The radioactivity contained in the precursor bands was quantitated. Semilogarithmic plots showed that the breakdown of pre-mAspAT follows first order kinetics (Fig. 2). The half-life of pre-mAspAT ranged from 4 to 6 min in different batches of CEF; however, it could be determined with a deviation of only $\pm 5\%$ from the mean value within the same batch. Methylamine was found to increase the half-life from 5.1 min in the control to 6.9 min which corresponds to an inhibi-

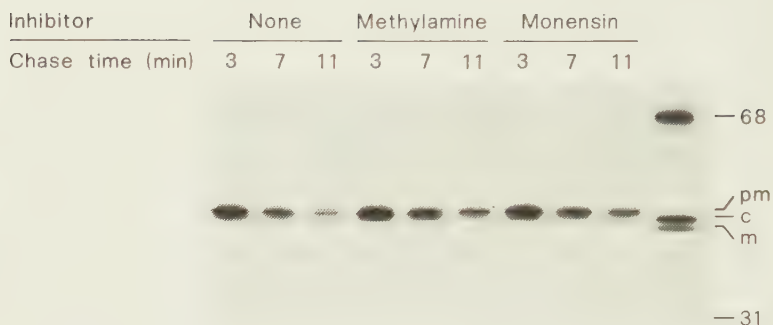


Fig. 1 Methylamine and monensin impair the decomposition of pre-mAspAT.

Growing CEF at high density were pulsed with [^{35}S]methionine and chased as described in "Experimental Procedures". The chase was carried out in the presence of 40 μM CCCP or 40 μM CCCP plus either 10 mM methylamine or 30 μM monensin. Pre-mAspAT was immunoprecipitated and analyzed with SDS-polyacrylamide gel electrophoresis and fluorography (for details, see "Experimental Procedures"). Pre-mAspAT (pm, $M_r = 47,500$) and the ^{14}C -labeled marker proteins (for sources, see Sonderegger et al., 1982) are indicated: bovine serum albumin (68), $M_r = 68,000$; cytosolic AspAT (c), $M_r = 46,000$; mAspAT (m), $M_r = 44,500$; carbonic anhydrase (31), $M_r = 31,000$.

tion by 26 %; monensin inhibited by 38 %. Similar results were obtained with 10 mM ammonium chloride (not shown).

The faint, poorly defined band running slightly faster than pre-mAspAT on SDS-polyacrylamide gel electrophoresis (see Figs. 1 and 3) was, independently of the inhibitors used, observed in most but not all cases. Excess unlabeled mAspAT prevented the immunoprecipitation of the band. Thus, this slightly lower-molecular weight material seems to originate from pre-mAspAT. Whenever present, the band disappeared with the same half-life as the precursor band. Conceivably, it represents intermediates of the precursor degradation. Alternatively, nonproteolytic

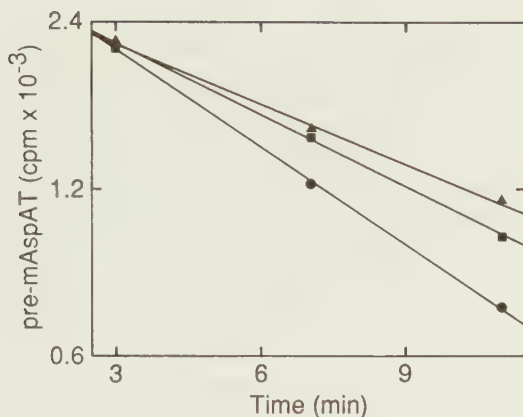


Fig. 2 **Determination of the half-life of pre-mAspAT.** The radioactivity contained in the pre-mAspAT bands of Fig. 1 was quantitated as described in "Experimental Procedures". $t_{1/2}$ of the precursor was calculated from the slopes of the regression lines and amounted to 5.1 min in the absence of lysosome-directed inhibitors (●), to 6.9 min in the presence of methylamine (■), and to 8.2 min in the presence of monensin (▲).

intracellular modifications or artifacts caused by experimental manipulations could give raise to the band.

In cells incubated in media containing serum, the rate of lysosomal proteolysis is slower than in cells depleted of serum (see "Discussion"). In order to assess the influence of serum on the degradation of pre-mAspAT, a pulse-chase experiment was carried out in the presence and absence of 10 % FCS. Serum was found to depress the precursor degradation by 41 % (Fig. 3).

The microbial protease inhibitory peptides leupeptin, antipain, and E-64 are known to act on intralysosomal proteolysis in a variety of cell types (for a review, see Seglen, 1983). Leupeptin strongly inhibits lysosomal and nonlysosomal cysteine

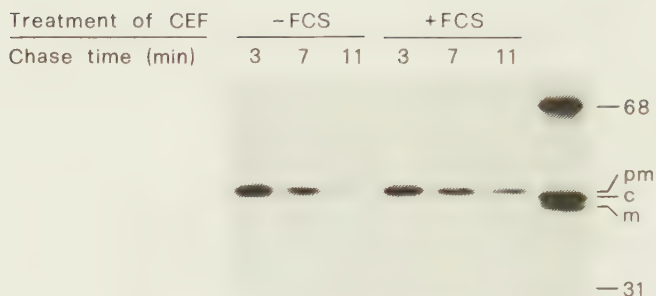


Fig. 3 **Effect of FCS on the proteolytic digestion of pre-mAspAT.** A pulse-chase experiment with CEF was performed in the absence (- FCS) or continuous presence (+ FCS) of 10% serum. The cells were pretreated with the respective media for 1 h prior to the chase and pulsed during the last 15 min of the pretreatment. Semilogarithmic plots (see "Experimental Procedures") indicated the half-life of the precursor to be 4.1 min in the absence and 6.9 min in the presence of FCS. Denotations of pre-mAspAT and marker proteins are as given in the legend to Fig. 1.

proteases as well as several serine proteases. Activity against the same protease classes, but with different relative effectiveness against the different enzymes, has been reported for antipain. Cysteine proteinases, especially cathepsin B, substantially contribute to the catabolism of proteins by lysosomes (Barrett, 1973; Huisman et al., 1973; Grinde and Seglen, 1980). Accordingly, the peptidic epoxide E-64 which irreversibly and specifically inhibits most cysteine proteinases has proven particularly effective in impeding lysosomal proteolysis. Leupeptin, antipain, and E-64 have all been found to inhibit extralysosomal proteolysis in cultured cells only to a negligible extent (Grinde and Seglen, 1980; Grinde, 1982; Gronostajski et al., 1984). In the present study, CEF were treated with a combination of leupeptin plus antipain or with E-64 for 1 h or 30 min, respectively, pulsed at the end of this treat-

ment, and finally chased in the presence of the inhibitors. In both cases, the rate of pre-mAspAT degradation was lowered by ~25 % (Table I). No further inhibition was brought forth by increasing the concentration of E-64 from 0.1 to 1 mM.

TABLE I Leupeptin, antipain, and E-64 affect pre-mAspAT degradation

Prior to be chased in the presence of the inhibitors, CEF were pretreated for 1 h with a combination of leupeptin and antipain, or for 30 min with E-64. Pulses were performed at the end of the pretreatments. The determination of the half-life of pre-mAspAT is described in "Experimental Procedures".

Inhibitor	Half-life of pre-mAspAT (min)	Inhibition of pre- mAspAT degradation (%)
None	5.4	0
Leupeptin/antipain (300 $\mu\text{g ml}^{-1}$ each)	7.1	24
E-64 (0.1 mM)	7.2	25

Additional evidence that only a minor part of pre-mAspAT is degraded by lysosomes came from experiments with compounds interfering with the cytoskeleton. Microtubuli are thought to be involved in the enhanced lysosomal uptake of proteins in cells maintained in media lacking serum, but not in the lysosomal catabolism taking place in the presence of serum (Amenta and Brocher, 1981). Treatment of CEF in the absence of FCS for 1.5 h with 50 μM of the microtubular poisons colchicine or podophyllotoxin resulted in a decrease of the rate of pre-mAspAT degradation by ~15 %.

3.2 Inhibitors of Intralysosomal Proteolysis and the Catabolism of Long-Lived CEF Proteins

The conclusion that ~30 % of pre-mAspAT are degraded in lysosomes can only be drawn from the observed ~30 % inhibition of precursor breakdown by lysosome-directed inhibitors if these agents inhibit lysosomal proteolysis completely. In order to check the extent of inhibition by lysosome-directed inhibitors, their effects on the proteolytic digestion of the preprotein were compared with those on the catabolism of long-lived proteins in CEF (for detailed explanation, see "Discussion"). The rate of degradation of long-lived proteins was determined by measuring the amount of trichloroacetic acid-soluble radioactivity released from this class of proteins during a chase. Long-lived proteins were specifically labeled by pulsing CEF for 16 h with [³⁵S]methionine and subsequent incubation for 2 h under chase conditions which allowed the degradative removal of labeled short-lived proteins. FCS depressed the catabolism of long-lived proteins during the following 4 h of chase from 9.2 to 6.2 % degradation (Table II), i.e. by 33 %. Similar values for both the rate of degradation and its depression by FCS have been found in rat embryo fibroblasts (Amenta and Brocher, 1980), human fibroblasts (Slot et al., 1986), and a 3T3 fibroblastic cell line (Gronostajski et al., 1984). Methylamine and a combination of leupeptin plus antipain inhibited the hydrolysis of long-lived proteins only slightly less than FCS. Thus, inhibitors of lysosomal function depress the catabolism of long-lived proteins by the same extent as that of pre-mAspAT (Table II). In the presence of FCS, the rate of degradation of long-lived proteins was not decreased by a combination of leupeptin plus antipain (not shown).

TABLE II Decrease of the breakdown of long-lived cellular proteins by inhibitors of intralysosomal proteolysis
The assay of the degradation rate of long-lived cellular proteins is specified in "Experimental Procedures". The mean values and the deviations of two experiments are indicated.

Treatment of CEF	Degradation of long-lived proteins (% degraded during 4-h chase)	Inhibition of degradation of long-lived proteins (%)	Inhibition of degradation of pre-maspAT proteins (%)
- FCS: control	9.2 ± 0.1	0	0
+ FCS (10 %)	6.2 ± 0.2	33	41 ^a
- FCS + methylamine (10 mM)	6.5 ± 0.2	29	26 ^a
- FCS + leupeptin/anti-pain (300 µg ml ⁻¹ each)	6.8 ± 0.1	26	24 ^b

^a Taken from the text of "Results".

^b Taken from Table I.

3.3 The Role of ATP in the Degradation of pre-mAspAT

Involvement of ATP in the proteolytic digestion of the precursor was experimentally demonstrated using CCCP and 2-deoxyglucose (dGlc) as agents interfering with the oxidative and the substrate level phosphorylation, respectively. The time dependence of ATP depletion of CEF with these reagents is presented in Fig. 4. The rate of precursor degradation was determined between 15 and 24 min after initiation of ATP depletion with 40 μ M CCCP plus 60 mM dGlc, i.e. at average ATP levels of 6 % and 89 % of the initial value in the depleted and the control

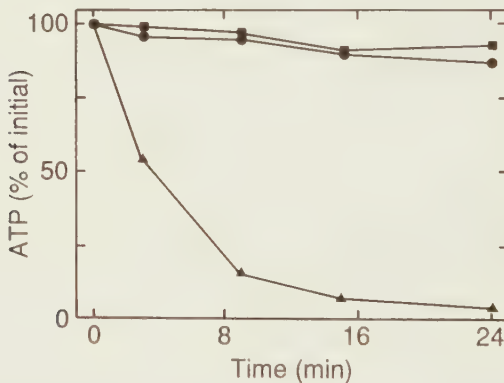


Fig. 4 Time dependence of ATP depletion. CEF were subjected to a mock pulse-chase experiment without [35 S]methionine. In addition to 40 μ M CCCP, the chase media contained no further ingredients (●), 60 mM dGlc (▲), or 60 mM sucrose (■). After the times indicated, the cultures were denatured, and ATP was measured as detailed in "Experimental Procedures". 100 % ATP correspond to $\sim$ 33 nmol ATP per Petri dish.

cells, respectively (Table III, experiment A). Under depletion conditions, proteolytic digestion of pre-mAspAT was diminished by 74 %. The effect of dGlc at 60 mM concentration is not due to the marked difference in the osmolarity of the culture medium. The same osmolarity difference brought about by sucrose

TABLE III ATP depletion and the breakdown of pre-mAspAT

Two different procedures for ATP depletion of CEF were used. A: Pulsed cells were incubated for 15 min in the chase media containing, in addition to 40 μ M CCCP, the compounds indicated. Subsequently, the rates of precursor degradation were determined during 9 min. The indicated values of the ATP content per Petri dish correspond to the mean of the values measured at 15 and 24 min (Fig. 4). B: A lower dose of dGlc than in experiment A was added. The incubation of CEF in the chase media lasted only 8 min and the precursor's half-life was measured during 10 min. The values for the ATP content correspond to the mean of the values measured at 8 and 18 min.

Additions to chase media	ATP content per Petri dish (% of initial)	Inhibition of pre- mAspAT breakdown (%)
A: None	89	0
dGlc (60 mM)	6	74
Sucrose (60 mM)	92	-11 ^a
B: None	90	0
dGlc (40 mM)	23	0

^a Breakdown stimulated by 11 %.

resulted in hardly any change in the ATP content of CEF (Fig. 4) and in a slight increase in the rate of preprotein degradation (Table III). When the ATP content of CEF averaged 23 % during the period of determination of the degradation rate, no change in the half-life of pre-mAspAT was observed (Table III, experiment B). Under the same conditions, the rate of protein synthesis decreased to 2 % of that measured in the control.

Inhibitors of various ATPases were investigated with respect to their effect on the degradation of pre-mAspAT. A diminution of the protein synthetic activity of CEF to <5 % of the control by treating the cells for 20 min with 1 mM vanadate or for 8 min with 0.5 mM quercetin proved both inhibitors to be effective in our cell cultures. Nevertheless, 1 mM vanadate lowered the precursor degradation under these conditions by only 15-20 % of the control and 0.5 mM quercetin did not inhibit at all. The slight decrease caused by vanadate may possibly be attributed to side effects of the compound such as inhibiting lysosomal cysteine proteinases (Seglen and Gordon, 1981).

3.4 Half-life of pre-mAspAT and Divalent Metal Ions

The charged metal chelator EDTA cannot easily permeate biological membranes and therefore does not rapidly enter cells. In order to deplete CEF selectively of divalent metal ions, we used the carboxylic ionophore A23187 in combination with EDTA as an extracellular ion trap. The degradation of pre-mAspAT was increasingly inhibited with increasing concentrations of A23187. A minimum concentration of 10 μ M of the ionophore was necessary in order to inhibit perceptibly the proteolytic digestion of pre-mAspAT whereas 90 % inhibition, i.e. 10 % of the degradative activity of the non-depleted control cells, required 60 μ M A23187 (Table IV). Reconstitution of the

TABLE IV Effect of divalent metal ion depletion and reconstitution on the degradation of pre-mAspAT in CEF

Pulsed CEF cultures were rinsed each with 3 ml BME that had been prepared without methionine, Mg^{2+} , and Ca^{2+} , and had been freed of divalent metal ions with Chelex 100 (Bio-Rad). The subsequent depletion of the cells was performed by supplementing the same kind of medium with A23187, EDTA, and 10 mM methionine. After 5 min, the BME used for depletion was removed, and a chase was carried out in order to determine the half-life of pre-mAspAT in the media with the specified additions. Metal ions were added as chlorides.

Treatment of CEF	Half-life of pre-mAspAT (min)	Rate of degradation of pre-mAspAT (% of control)
None	4.5	100
a: A23187 (60 μM)/ EDTA (0.5 mM)	44.6	10
a + Mg^{2+} (1.5 mM)	4.2	107
a + Ca^{2+} (1.5 mM)	19.1	24
a + Zn^{2+} (1.5 mM)	>1000	0

depleted CEF with Mg^{2+} 1 mM in excess to EDTA fully restored the rate of pre-mAspAT degradation. In contrast, Ca^{2+} augmented the degradation rate from 10 % in the absence of reconstituting ions to only 24 %, and Zn^{2+} , a prosthetic group of a great number of metalloproteases, even abolished the degradation of pre-mAspAT. The difference in the capacities of Mg^{2+} and Ca^{2+} to stimulate the breakdown of pre-mAspAT is unlikely to be due to differences in transport activities of A23187; the affinities of A23187 for Mg^{2+} and Ca^{2+} are almost the same (Pfeiffer

et al., 1974). 1,10-Phenanthroline that enters cells and strongly complexes Zn^{2+} but binds Mg^{2+} and Ca^{2+} very weakly (Martell and Smith, 1977) pronouncedly inhibited the degradation of the precursor only at a concentration of 5-10 mM (Jaussi et al., 1984, and unpublished data). Apparently, pre-mAspAT in CEF is not attacked by Zn^{2+} -dependent but rather, at least in part, by cysteine proteases, enzymes which often are inhibited by Zn^{2+} (Barrett, 1973; Ismail and Gevers, 1983). The additional inhibition of the proteolysis of the preprotein brought about by Zn^{2+} in high concentration might also be attributed to more general perturbations of the cellular metabolism connected to the severe cytotoxicity of the ion (National Research Council, Subcommittee on Zinc, 1979) rather than to inhibition of cysteine proteases.

Additional evidence that Ca^{2+} is not essential for the catabolism of pre-mAspAT was obtained from experiments with the synthetic neutral ionophore ETH 1001. This ion ligand has an extremely high selectivity for Ca^{2+} over Mg^{2+} (Simon et al., 1978) and mediates Ca^{2+} flux across biological membranes without promoting the movement of Mg^{2+} (Hinds and Vincenzi, 1985). Depleting CEF of Ca^{2+} with 100 μM ETH 1001 plus the specific calcium chelator ethyleneglycol-bis-(3-aminoethyl ether)-N,N,N',N'-tetraacetic acid (0.5 mM) in a pulse-chase experiment analogous to that described in the legend to Table IV diminished the breakdown of the precursor only slightly (not shown).

Ca^{2+} -supplemented eucaryotic cells incorporate amino acids into proteins up to 10 times more rapidly than Ca^{2+} -depleted cells, with Mg^{2+} facilitating the stimulation by Ca^{2+} (Brostrom et al., 1983). Correspondingly, the rate of protein synthesis was decreased by ~95 % in CEF that had been depleted of divalent metal ions with 60 μM A23187 plus 0.5 mM EDTA for 5 min. As

mentioned above, protein synthesis collapsed also in CEF depleted of ATP or treated with the ATPase inhibitors vanadate or quercetin. However, a decrease in the rate of protein synthesis of 99 % brought about by treating CEF with 1 mM cycloheximide did not alter the rate of pre-mAspAT decomposition.

3.5 Response of pre-mAspAT Breakdown to Various Inhibitors

Modification of sulfhydryl groups by iodoacetamide, N-ethylmaleimide, and p-hydroxymercuribenzoate led to complete inhibition of the degradation of the precursor (Table V), suggesting that both the lysosomal and extralysosomal degradation of the preprotein depend on the integrity of certain sulfhydryl groups. In contrast, the serine protease inhibitors, phenylmethylsulfonyl fluoride and p-aminobenzamidine, inhibited only slightly or not at all, respectively. Na-p-Tosyl-L-lysine chloromethyl ketone partially inhibits the decomposition of the precursor and, probably linked to a covalent modification of pre-mAspAT, effects a slight increase in the precursor's apparent molecular weight (Jaussi et al., 1984). Similar effects on pre-mAspAT have been observed with L-1-tosylamido-2-phenylethyl chloromethyl ketone (R. Jaussi, personal communication). The bacillar polypeptide bacitracin inhibits a broad spectrum of proteases (Mäkinen, 1972) and has been shown to be effective in different types of intact cells (Roth, 1981). Bacitracin lowered the breakdown of pre-mAspAT by 42 %. Bestatin, isolated from a *Streptomyces* strain as a potent competitive inhibitor of leucine aminopeptidase and aminopeptidase B (Suda et al., 1976), did not diminish the rate of pre-mAspAT breakdown.

Rhodamine 123 at low doses is a specific fluorescent dye for mitochondria (Johnson et al., 1980). The maturation of several mitochondrial precursor proteins is reported to be inhibited

TABLE V Influence of inhibitors of various classes of proteases on the degradation of pre-mAspAT

Before starting the chases for determining the half-life of pre-mAspAT in the presence of the indicated inhibitors, the cells were pretreated with the inhibitors iodoacetamide, N-ethylmaleimide, and p-hydroxymercuribenzoate for 5 min, with phenylmethylsulfonyl fluoride, p-aminobenzamidine, and bacitracin for 60 min, and with bestatin for 40 min.

Inhibitor	Inhibition of pre-mAspAT degradation (%)
Iodoacetamide (5 mM)	95
N-Ethylmaleimide (5 mM)	94
p-Hydroxymercuribenzoate (1 mM)	96
Phenylmethylsulfonyl fluoride (2 mM)	23
p-Aminobenzamidine (2 mM)	0
Bacitracin (5 mM)	42
Bestatin (200 $\mu\text{g ml}^{-1}$)	0

under rhodamine 123 (Anderson, 1981). In living CEF, rhodamine 123 inhibited both the maturation and the catabolism of pre-mAspAT almost completely (not shown). However, the concentration of rhodamine 123 needed for a clear effect in our system was 0.6 mM and thus exceeded by far the concentrations under which the effect of rhodamine 123 is restricted to mitochondria (James and Bohman, 1981). The observed inhibition of pre-mAspAT maturation and degradation therefore does not necessarily re-

flect specific interactions between rhodamine 123 and mitochondria. The results of experiments with rhodamine B also argue against a participation of mitochondria in the catabolism of pre-mAspAT. The neutral rhodamine B, in contrast to the positively charged rhodamine 123, does not specifically interact with mitochondria (Johnson et al., 1980). Yet, 0.6 mM rhodamine B decreased the preprotein maturation and degradation to the same extent as 0.6 mM rhodamine 123.

3.6 Degradation of pre-mAspAT in Crude CEF Extracts and in Permeabilized CEF

Harvesting and homogenizing the cells by mechanical means prolonged the precursor's half-life up to 2 h (Jaussi et al., 1984). The capacity of intact CEF to degrade pre-mAspAT could not be preserved on homogenization even though the experimental conditions of the preparation of the cell extracts and the conditions for their subsequent incubation were widely varied. The residual degradative activity in crude extracts did not depend on ATP and Mg^{2+} . Thus, the slow breakdown of the precursor in homogenates is unlikely to be due to restricted activity of the same proteases that attack pre-mAspAT in intact CEF. The reasons for the prolongation of the precursor's half-life in crude CEF extracts are unknown. Conceivably, the preparation of crude extracts leads either to the destruction of labile proteases digesting pre-mAspAT in intact CEF, or to interactions of the proteases in question with endogenous inhibitors, or to competitive inhibition of the precursor degradation by protease substrates released on homogenization. Loss of activity of intracellular proteolytic systems on cell homogenization has frequently been noticed (Huisman et al., 1974; Rose et al., 1979; Hathaway et al., 1982; Waxman et al., 1985).

Digitonin permeabilization of the cells was performed as an alternative to cell homogenization. It was found that 0.003 % digitonin, the lowest concentration which still resulted in trypan blue stainability of >95 % of CEF, almost completely depressed the degradation of the preprotein. Only 5-10 % of total pre-mAspAT were released from CEF by treatment with 0.003 % digitonin; apparently, leakage of the precursor does not account for the detrimental effect of digitonin on the degradation rate.

4. DISCUSSION

4.1 Contribution of Lysosomes to the Breakdown of pre-mAspAT

Intracellular protein degradation proceeds in several different compartments with the relative importance of each of these degradative pathways depending on the particular cell type and its metabolic state. The lysosomes are the only cellular compartment equipped to catalyze the complete hydrolysis of most cell proteins. The contribution of lysosomes to the degradation of pre-mAspAT was determined by treating growing CEF with inhibitors of lysosomal proteolysis. Members of four mechanistically different classes of lysosome-directed inhibitors, i.e. the lysosomotropic amines methylamine and ammonium chloride, the carboxylic ionophore monensin, FCS, as well as the protease inhibitors leupeptin, antipain, and E-64 were all found to diminish the rate of pre-mAspAT breakdown by $\sim 30\%$. The question was raised whether this partial inhibition reflected a partition of the degradation of the precursor between the lysosomal and extralysosomal compartment or whether it was due to an only partial inhibition of lysosomal activity. In order to estimate the extent of inhibition of lysosomal proteolysis achieved with the above inhibitors, their effect on the catabolic rate of long-lived proteins in CEF was determined. FCS withdrawal from cell cultures selectively stimulates the breakdown of long-lived cellular proteins whereas the breakdown of short-lived proteins remains unchanged (Amenta et al., 1980; Neff et al., 1981; Gronostajski et al., 1984). This stimulation is solely due to an increase in the lysosomal activity (Amenta et al., 1978, 1980; Gronostajski et al., 1984, 1985). FCS depletion of CEF stimulated the breakdown of long-lived proteins by a factor of ~ 1.5 . For ease of comparison, the stimulation by FCS depletion is expressed as per cent inhibition by FCS (see

Table II). The lysosomotropic weak base methylamine and a mixture consisting of the peptidic cathepsin inhibitors leupeptin plus antipain impeded the stimulated breakdown of long-lived proteins by nearly the same degree as FCS. The lysosomotropic weak bases and the peptidic inhibitors affect intralysosomal proteolysis both under serum deprivation and under nutritionally complete, so-called basal conditions (Amenta and Brocher, 1981). However, the basal rate of lysosomal proteolysis in growing fibroblasts amounts to only a small fraction of that under stimulation by FCS depletion (Amenta et al., 1980; Gronostajski et al., 1984, 1985). Accordingly, in the presence of FCS, the inhibition of the degradation of long-lived proteins by a combination of leupeptin plus antipain proved to be negligible. Thus, the inhibition of lysosomal proteolysis by the lysosome-directed inhibitors is almost complete. Consequently, the ~30 % inhibition of pre-mAspAT degradation indicate that in the absence of FCS ~30 % of pre-mAspAT are degraded by lysosomes. The small basal rate of lysosomal proteolysis implies that, in the presence of FCS, much less than ~30 % of pre-mAspAT are broken down by lysosomes.

Remarkably, the stimulation of the precursor degradation by FCS withdrawal (Fig. 3) does not agree with the generally accepted rule that depletion of FCS stimulates lysosomal degradation specifically of long-lived proteins, while, in growing cells under basal conditions, both short- and long-lived proteins are extralysosomally catabolized. Our data rather suggest that pre-mAspAT and long-lived proteins share structural features which are recognized by the stimulated lysosomal system (Rivett, 1986).

4.2 Localization of the Protease(s) Responsible for the Degradation of the Bulk of pre-mAspAT

It seems very unlikely that pre-mAspAT accumulated in the cytosol under conditions of import block is taken up and degraded by organelles other than lysosomes. The precursor not digested in lysosomes is most probably degraded by soluble cytosolic proteases or by proteases on the cytosolic surfaces of cellular membranes. As reported previously, the protease inhibitor p-aminobenzamidine possibly causes intramitochondrial degradation of mitochondrial preproteins (Raymond and Shore, 1981). The precursor of the mitochondrial matrix enzyme carbamoyl-phosphate synthetase was found to accumulate in the cytosol of rat liver explants and to get degraded with a half-life of 2-3 min when its processing was inhibited with p-aminobenzamidine. p-Aminobenzamidine was presumed not to block the uptake of pre-carbamoyl phosphate synthetase but its maturation by the processing proteinase in the mitochondrial matrix. Accordingly, pre-carbamoyl phosphate synthetase was postulated to get broken down inside the mitochondria. The question of an involvement of cytosolic or lysosomal proteases was not studied. In contrast to p-aminobenzamidine, CCCP is known to block the uptake of mitochondrial precursors. Thus, intramitochondrial degradation of pre-mAspAT in CEF treated with CCCP can be excluded. However, degradation of pre-mAspAT on the outer membranes of mitochondria cannot be excluded, for CCCP does not interfere with the binding of preproteins to mitochondria (Schleyer et al., 1982).

4.3 The Roles of ATP, Mg^{2+} , and Ca^{2+} in pre-mAspAT Degradation

In CEF depleted of divalent metal ions or ATP, the rate of pre-mAspAT breakdown was reduced by ~90 % (Table IV) or ~70 %

(Table III), respectively. Metal depletion obviously inhibits both the cytosolic (~70 % of total) and the intralysosomal (~30 % of total) degradation of the precursor almost completely. ATP depletion also affects most probably both the lysosomal and the cytosolic pathway. Deceleration of the degradation of pre-mAspAT by ~70 % required a reduction of cellular ATP to less than 10 % of the control whereas the breakdown of the preprotein was not affected if ATP was reduced to only 23 % (Table III) even though protein synthesis collapsed under these conditions. The relative insensitivity of precursor degradation toward ATP depletion indicates a high affinity of the responsible proteolytic systems for ATP.

Intracellular breakdown of both short- and long-lived proteins in growing fibroblasts largely depends on ATP as an energy source (Gronostajski et al., 1985). Especially in enzymatic reactions involving transfer of phosphate groups of ATP, Mg^{2+} is usually required as a cofactor. Probably, Mg^{2+} confers full activity in pre-mAspAT degradation to metal depleted CEF (Table IV) by re-forming the $Mg \cdot ATP$ complex possibly followed by hydrolysis of ATP. Lysosomal proteolysis is sustained by $Mg \cdot ATP$ in at least two ways. First, acidification of lysosomes resulting in optimum conditions for cathepsins is chiefly driven by a $Mg \cdot ATP$ -dependent ATPase in the lysosomal membrane (Mellman et al., 1986), and second, uptake of proteins into lysosomes as stimulated by FCS depletion has been reported to be connected to microtubular functions (Amenta and Brocher, 1981; Grinde, 1985) which, in turn, depend on $Mg \cdot ATP$.

Cytosolic proteases dependent on $Mg \cdot ATP$ have also been reported (see below). Requirement of $Mg \cdot ATP$ for intracellular proteolysis is thus a well known phenomenon. If $Mg \cdot ATP$ -dependent breakdown of pre-mAspAT were followed by hydrolysis of ATP, vanadate and quercetin, inhibitors of various soluble and membrane-bound

ATPases, might decelerate the precursor degradation. Yet, no or little inhibition by vanadate or quercetin was observed and thus it remains an open question whether the degradation of pre-mAspAT is connected to hydrolysis of ATP.

Although Ca^{2+} is necessary for continuous autophagy (Grinde, 1983), reconstitution with Mg^{2+} alone fully restored the rate of pre-mAspAT degradation in CEF depleted of divalent metal ions. Reconstitution with Ca^{2+} had little effect. A much greater importance of Mg^{2+} than of Ca^{2+} has also been shown for lysosomal proteolysis in hepatocytes (Grinde, 1983). Residual Ca^{2+} remaining after metal deprivation and available to the autophagic apparatus might suffice to sustain autophagy of pre-mAspAT in CEF reconstituted with Mg^{2+} alone. Alternatively, Mg^{2+} might liberate Ca^{2+} from intracellular pools (Nemeth and Scarpa, 1986) originally not available to autophagy. Inhibition of pre-mAspAT degradation cannot be explained in terms of the collapse of protein synthesis in CEF depleted of ATP or divalent metal ions, as virtually complete inhibition of protein synthesis achieved with cycloheximide did not alter the rate of precursor degradation.

4.4 Are Previously Described Cytosolic Proteases Involved in the Degradation of pre-mAspAT?

The peptidic inhibitors leupeptin, antipain, and E-64 strongly depress most lysosomal and nonlysosomal cysteine proteases (see above). Nevertheless, all three compounds decreased the rate of pre-mAspAT degradation in CEF by only ~30 % (Table I). If, in addition to intralysosomal cysteine proteases, cytosolic cysteine proteases were involved in the degradation of the precursor, the observed inhibition would be expected to exceed this value. Although cysteine proteases apparently are not respon-

sible for the cytosolic breakdown of the preprotein, modification of thiol groups with iodoacetamide, N-ethylmaleimide, or p-hydroxymercuribenzoate resulted in almost complete inhibition of pre-mAspAT degradation. Thus, pre-mAspAT seems to be attacked by one or several cytosolic proteases dependent on thiol groups that, however, might serve another function than the thiol groups at the active sites of known cysteine proteases. The thiol groups essential for precursor breakdown might be localized either on the proteases themselves, or on pre-mAspAT, or on hypothetical regulatory factors.

In recent years, numerous high molecular weight cytosolic proteases with pH optima in the neutral to alkaline range have been identified in a variety of mammalian cells and tissues. The relationship among the various high molecular weight cytosolic proteases, however, has to date remained unclear and no such enzymes have so far been isolated from non-mammalian cells. The inhibitor characteristics of the high molecular weight protease found in the cytosol of undifferentiated murine erythroleukemia cells (Waxman et al., 1985) are strikingly similar to the characteristics of extralysosomal pre-mAspAT degradation: The protease is strongly inhibited by thiol reagents, only slightly affected by serine protease inhibitors, and not at all by peptidic inhibitors of cysteine proteases. It is stimulated by ATP, but only in the presence of Mg^{2+} . ATP-stimulated proteolytic activity was found in crude extracts of murine erythroleukemia cells but not of fibroblasts although intact fibroblasts are known to possess an ATP-dependent nonlysosomal proteolytic route for the degradation of short- and long-lived proteins (Gronostajski et al., 1985, and this paper). The breakdown of pre-mAspAT also corresponds with this last feature, as the rapid degradation of the preprotein was not observed in crude extracts of CEF. High molecular weight proteases other than that found in murine erythroleukemia cells

differ in some respects from the protease(s) responsible for the extralysosomal breakdown of pre-mAspAT. ATP has been reported to stimulate degradation by these enzymes to variable degrees in several instances (DeMartino and Goldberg, 1979; Rose et al., 1979; Ismail and Gevers, 1983), but brought about no stimulation in other cases (Ishiura et al., 1985; Rivett, 1985; Tanaka et al., 1986). Mg^{2+} has proven either not to be necessary (Rose et al., 1979; Rivett, 1985; Ishiura and Sugita, 1986) or even to hamper the proteases (DeMartino and Goldberg, 1979; Ishiura et al., 1985; Yamamoto et al., 1986). Furthermore, peptidic inhibitors of cysteine proteases repeatedly have been found to depress the catalytic activity of certain high molecular weight cytosolic proteases (Ishiura et al., 1985; Rivett, 1985; Yamamoto et al., 1986).

A multi-enzyme proteolytic system dependent on Mg-ATP and covalent labeling of its substrate proteins with the polypeptide ubiquitin has originally been discovered in the cytosol of reticulocytes (for a recent review, see Finley and Varshavsky, 1985). It is unlikely that this system acts upon pre-mAspAT in the cytosol of CEF, as 1 mM vanadate depressed the breakdown of endogenous ubiquitin-conjugated proteins in reticulocytes by 80-90 % (Tanaka et al., 1984) but inhibited pre-mAspAT degradation in CEF only by 15-20 %. Moreover, 0.1 mM quercetin decreased the activity of the ubiquitin-dependent system in cell homogenates of reticulocytes by ~30 % (Tanaka et al., 1984) whereas 0.5 mM quercetin led to no deceleration of precursor catabolism in CEF. In contrast to the ubiquitin-dependent system which is active in crude cell extracts, the degradation of pre-mAspAT virtually ceases on cell homogenization. Finally, an ATP-dependent proteolytic activity has not been found in crude extracts of fibroblasts (Waxman et al., 1985).

The results of the reconstitution experiments with CEF depleted of divalent metal ions suggest that neither Ca^{2+} - nor Zn^{2+} -activated proteases attack pre-mAspAT. An extensively studied group of extralysosomal metalloproteases are the Ca^{2+} -dependent neutral proteases localized predominantly in the cytosol of a great number of mammalian and avian cell types (Murachi, 1983). Ca^{2+} -dependent neutral proteases are not activated by Mg^{2+} (Suzuki and Ishiura, 1983), do not respond to ATP, and show strongly lowered activity in the presence of peptidic inhibitors of cysteine proteases (Ishiura et al., 1978; Siman et al., 1984), each of these features contrasting the cytosolic degradation of pre-mAspAT.

The degradation of the bulk of pre-mAspAT accumulated in the cytosol of CEF on treatment with CCCP thus appears to be due to one or several cytosolic proteases dependent on ATP, Mg^{2+} , and thiol groups of unknown localization, conceivably to proteases identical with or similar to high molecular weight cytosolic proteases (Waxman et al., 1985).

4.5 Physiological Significance of pre-mAspAT Degradation

The import of pre-mAspAT into mitochondria ($t_{1/2}$ 0.5 - 1 min) is a much faster process than the degradation of the preprotein ($t_{1/2} \sim 5$ min). Therefore, the rate of degradation could only be determined under conditions of import block, leaving the possibility that the observed degradation might be restricted to such conditions. However, pre-mAspAT accumulated by treating CEF for 75 min with CCCP is quantitatively imported into mitochondria when cysteamine, an antagonist of CCCP, is added in order to restore the mitochondrial membrane potential (Jaussi et al., 1982). Obviously, accumulated pre-mAspAT retains a structure which allows recognition, uptake, and processing by

mitochondria. Thus, rapid breakdown of accumulated preprotein does not seem to be linked to structural changes of aged pre-mAspAT but rather can be assumed to proceed also in cells not treated with uncouplers.

Breakdown of pre-mAspAT by one or several enzymes similar to high molecular weight cytosolic proteases and by lysosomes would imply that cells are not equipped with a proteolytic system specific for precursors of nuclear-encoded mitochondrial proteins. These preproteins rather are attacked by multifunctional proteolytic systems. The fast rate of degradation of pre-mAspAT thus appears to be based on intrinsic structural features of the preprotein rather than on a particularly effective catabolic pathway.

The physiological relevance of proteolytic removal of precursors of mitochondrial proteins that are synthesized in the cytosol is presently unknown. Conceivably, intracellular proteolytic systems might exert a regulatory function in mitochondrial assembly and development (see "Introduction"), such as has been suggested for cellular proteases in yeast (Luzikov et al., 1976) and for a proteolytic pathway found in rat liver mitochondria (Desautels and Goldberg, 1982).

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